Palaeophytochemical Components from the Miocene-Fossil Wood of *Pinus griffithii*

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Some Miocene-fossil wood of *Pinus griffithii* preserved as lignified wood in brown coal was found in an open coalmine in Xundian of Yunnan Province, China. To explore its chemical components, here we show the palaeophytochemical investigation of this Pliocene-fossil wood of *P. armandii*, resulting in the isolation of 11 compounds (**1**-**11**) including one new compound named 3,3-dimethoxy-24-ethyl-cholestan (**1**) by liquid column chromatography. Furthermore, sixteen volatiles were detected from this fossil wood by gas chromatography-mass spectrometry (GC-MS). These structures of 11 compounds were elucidated by analysis of their MS, 1D and 2D-NMR spectra, and comparison with published data.

Keywords: Pinus griffithii; Miocene-fossil; 3,3-Dimethoxy-24-ethyl-cholestan; Chemical components.

INTRODUCTION

The analysis of chemical constituents in specific plant fossils provides crucial information for their probable diagenetic pathways. Natural products in plant fossils could retain their characteristic basic structural skeletons and be used as chemosystematic marker or biomarkers for their biological origin, though they may undergo rapid diagenetic processe during their fossilization.[1,2] Some natural flavonoids, steroids, and other constituents were detected from Jurassic, Cretaceous, Pliocene, Miocene and Eocene plants, previously.[3-9] *Pinus griffithii* is an economically important conifer indigenous to the southwest and center regions of China.[10] A piece of Miocene-fossil wood of *P. griffithii*, which was preserved morphologically, was discovered in an open Jinsuo coalmine in Xundian of Yunnan Province, China, raising questions about its phytochemical constituents. In this paper, we carried out the palaeophytochemical investigation by phytochemical methods including gas chromatography-mass spectrometry (GC-MS) and liquid column chromatography.

RESULTS AND DISCUSSION

An ethanol extract prepared from the Miocene-fossil wood of *P. griffithii* was purified repeatedly by column chromatography (CC) on silica gel and Sephadex LH-20 and then subjected to preparative thin-layer chromatography, yielding a new steroid derivative (**1**) and other ten known compounds (**2-11**) as showed in Fig. 1.

Compound **1** was obtained as colorless needles. Its molecular formula $C_{31}H_{56}O_2$, with four unsaturation degrees, was deduced from the quasi-molecular ion peak at *m/z* 460 in its EI mass spectrum and the ^{13}C-NMR (DEPT) spectrum, which was supported by its HR-ESI observed at *m/z* 483.4164 (calcd. 483.4178, $[M + Na]^+$). The ^{1}H-NMR spectrum of compound **1** showed the characteristic signals of six methyl groups at δ 0.9 (d, J = 5.2 Hz), 0.82 (d, J = 5.6 Hz), 0.80 (d, J = 5.6 Hz), 0.84 (d, J = 6.4 Hz), 0.78 (s) and 0.64 (s). Comparison with the ^{1}H-NMR spectral data in the literature[5] suggested that **1** had a typical steroid skeleton. Additionally, two methoxy group signals at δ 3.14 (s) and 3.19 (s) were shown in the ^{1}H-NMR spectrum. The ^{13}C-NMR and DEPT spectrum of compound **1** revealed the presence of thirty-one carbon atoms including two methoxy groups. The ^{13}C-NMR spectral data (Table 1) was similar with stigmastan-3-one (**5**) except for the substitution of C-3 whose chemical shift of C-3 was highfield shifted to δ

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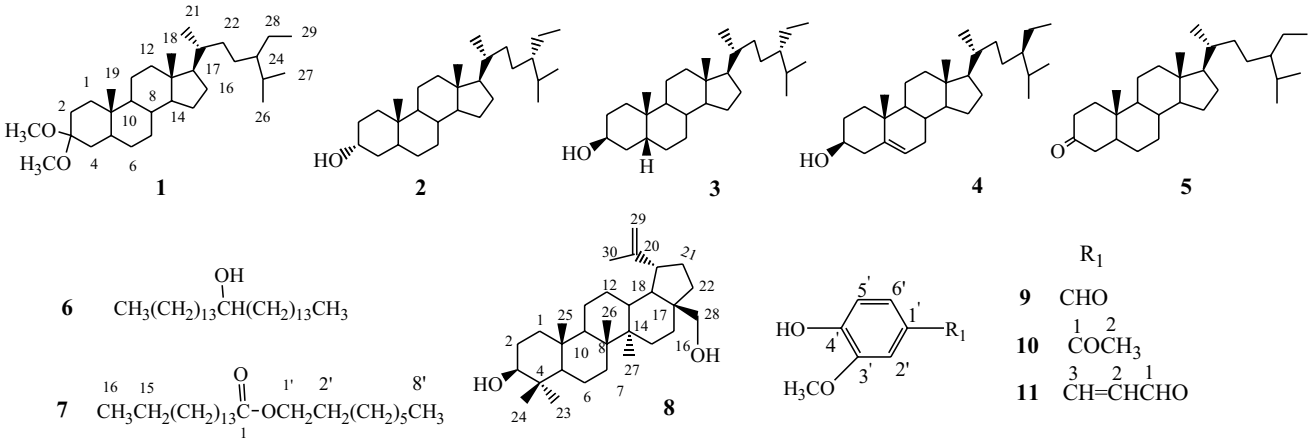

Fig. 1. Structures of compounds **1-11**.

100.4 (s), suggesting that C-3 was substituted by two methoxy groups. It was supported by the HMBC spectrum (Fig. 2), exhibiting the correlations of the methoxy protons at δ 3.14 (s) and δ 3.19 (s) with C-3 (δ 100.4). Moreover, EI mass spectrum displayed the fragment ion peak at *m/z* 429, corresponding to the loss of one methoxy group from the molecular ion peak at *m/z* 460. The assignment of **1** was further supported by its ^{1}H-^{1}H COSY and HMQC spectrum. Based on the above spectral evidence, compound **1**, a new steroid, was identified as 3,3-dimethoxy-24-ethyl-cholestan.

Compound **1** isolated from this Miocene-fossil wood of *P. griffithii* here might be its inner natural product or chemical transformed ones from compound **5** by acid media derived from environment such as coals and sediments. Moreover, ten known compounds (**2**-**11**) were also isolated from the Miocene-fossil wood of *P. griffithii*. Their structures were elucidated to be 24α-ethyl-cholestan-3α-ol (**2**),[11,12] 5β-24S-ethyl-cholestan-3β-ol (**3**),[13,14] 3β-sitosterol (**4**),[5] stigmastan-3-one (**5**),[5] 15-nonacosanol (**6**),[15] octyl palmitate (**7**),[16] 20(29)-lupene-3,28-diol (**8**),[17,18] vanillin (**9**),[19] acetovanillone (**10**),[20] and coniferaldehyde (**11**),[21,22] according to the analysis of their spectral data and literature, respectively.

Three steroids (**2**, **4** and **5**) were previously isolated from Pliocene-fossil wood.[5] The steroid skeleton has been

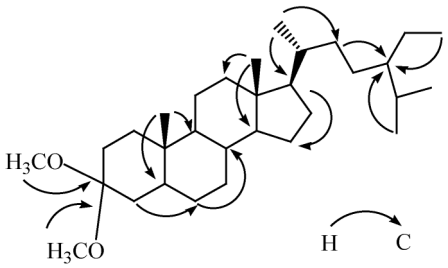

Fig. 2. Selected HMBC correlations of **1**.

Table 1. ^{13}C-NMR data (100 MHz, in $CDCl_3$) of compounds **1** and **5**

No.	**1**	**5**	No.	**1**	**5**	No.	**1**	**5**
1	35.0 t	38.5 t	11	21.2 t	21.4 t	21	18.7 q	18.7 q
2	28.5 t	38.2 t	12	40.0 t	39.9 t	22	33.9 t	33.9 t
3	100.4 s	212.3 s	13	42.6 s	42.6 s	23	26.0 t	26.0 t
4	35.6 t	44.7 t	14	56.1 d	56.1 d	24	45.8 d	45.8 d
5	42.4 d	46.7 d	15	24.2 t	24.2 t	25	29.1 d	29.1 d
6	28.3 t	29.0 t	16	28.3 t	28.2 t	26	19.8 q	19.8 q
7	32.0 t	31.7 t	17	56.4 d	56.3 d	27	19.0 q	19.0 q
8	35.5 d	35.4 d	18	12.0 q	12.0 q	28	23.0 t	23.0 t
9	54.0 d	53.8 d	19	11.6 q	11.4 q	29	12.0 q	11.9 q
10	35.7 s	35.6 s	20	36.2 d	36.1 d	$2OCH_3$	47.5 q	-

widely distributed in environmental sediments and used as an indicator of diagenesis and biomarker of organic matter.[23-25] These steroids isolated in fossil wood may be derived from environmental sediments. Phenolic compounds **9**-**11**, also isolated from Miocene fossil wood of *Picea likiangensis*,[22] may be the degradation products of lignans[26] derived from sediments, 3β-sitosterol can effectively scavenge hydroxyl radical and superoxide anion radical which indicated the potential value as oil antioxidant.[27] Vanillin can inhibit the activities of both monophenolase and diphenolase.[28] Coniferaldehyde could stimulate UMR106 cell proliferation and exhibit weak antioxidant activity.[29,30] Sixteen volatiles (Table 2) from Miocene fossil wood of *P. griffithii* were detected by GC-MS analysis, including alkanes (C_{12}~C_{17}), alkanols, naphthalenes, terpenoids, aromatic compounds and sterols. Phytochemical components analyzed from the Miocene-fossil wood of *Pinus griffithii* indicated that these chemical components were well-preserved though some natural products altered and transferred during the period of burial in sediments.

EXPERIMENTAL

General experimental procedures

Melting points were measured on an XRC-1 micro-melting apparatus and are uncorrected. IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV spectra were recorded on a UV 210A spectrometer. MS spectra were carried out on a VG Auto Spec-3000 spectrometer. The 1D and 2D NMR spectra were run on Bruker AM-400 MHz and DRX-500 MHz spectrometer using TMS as an internal. Silica gel (200-300 mesh, Marine Chemical Factory, Qingdao, China) were used for column chromatography.

Plant material

The Miocene-fossil wood of *Pinus griffithii* was collected from an open Jinsuo coalmine in Xundian of Yunnan Province, People's Republic of China. The identity of this fossil wood material was verified by professor Cheng-Sen Li and a voucher specimen (KMJS-01) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Analysis of volatiles

The powdered Miocene-lignified wood of *P. griffithii* (12 g) was extracted for 4 × 3 h with hot methanol. The extract was concentrated in vacuum at room temperature to give the residue, which were partitioned between n-hexane and methanol to give the n-hexane layer and methanol layer, respectively. The n-hexane extracts (40 mg) was subjected to GC-MS analysis with Agilent HP6890GC/

Table 2. Volatiles from the Miocene fossil wood of *Pinus griffithii*

No.	Retention Time (min)	Compounds	Molecular Formula	Relative Content (%)
1	7.33	Dodecane	$C_{12}H_{26}$	0.18
2	10.00	1-Methylnaphthalene	$C_{11}H_{10}O$	0.24
3	12.73	1,5-Dimethylnaphthalene	$C_{12}H_{12}O$	0.18
4	12.92	2,4-Dichloro-benzoic acid, methyl ester	$C_8H_6O_2Cl_2$	0.14
5	15.22	γ-Cadinene	$C_{15}H_{24}$	0.62
6	17.09	Hexadecane	$C_{16}H_{34}$	0.34
7	17.47	α-Cedrol	$C_{15}H_{26}O$	5.66
8	19.43	Heptadecane	$C_{17}H_{36}$	0.34
9	23.36	2-Methylthio-4-(2-styrylcyclopropyl)thiophene	$C_{16}H_{16}S_2$	4.28
10	23.49	6-Methoxy-2,3,3,9-tetramethyl-2,3-dihydro-4H-furo[3,2-c][1]benzpyran-4-one	$C_{16}H_{18}O_4$	1.15
11	24.88	(-)-Kaurene	$C_{20}H_{32}$	1.41
12	25.75	11-Methyl-6H-pyrido[4,3-b]carbazole-5-methanol	$C_{17}H_{14}N_2O$	8.33
13	26.04	7-Ethyltetradecahydro-1,1,4a,7-tetramethyl-phenanthrene	$C_{20}H_{36}$	11.43
14	26.61	2-Methyl-4-(4′-methoxyphenyl)-6-(2′-pyridyl)-pyridine	$C_{18}H_{16}N_2O$	1.64
15	33.71	4,14-Dimethyl-9,19-cycloergost-24(28)-en-3-ol	$C_{30}H_{50}O$	1.66
16	35.12	3β-9,19-Cyclolanost-24-en-3-ol	$C_{30}H_{50}O$	1.87

5973MS instruments. The GC was operated as follows: analysis column, HP-5MS (30 m × 0.25 mm × 0.25 μm); column temperature, held from 80 °C and ramped at 3 °C min^{-1} to 240 °C; elution, pure N_2; concentration of n-hexane extract, 10 mg/mL and injection volume, 0.1 μL. The MS was operated in full scan mode (35-450, 1 scan/s, 70 eV ionization energy). Peaks were identified by comparison of their relative retention times, and mass spectral characteristics, with NBS mass spectral library software and relative literatures.

Extraction and isolation

The powdered Miocene-lignified wood of *P. griffithii* (5.0 Kg) was extracted with hot 95% EtOH (10 L × 3) and filtered. The ethanol extraction was evaporated in vacuum to give the residue. The residue (150 g) was subjected to column chromatography (CC) on silica gel (200-300 mesh), eluted with petroleum/acetone (4:1) to yield 4 fractions. Fraction 1 (52 g) was purified by repeated CC (silica gel with petroleum/acetone 3:1 and 8:1) and then CC (Sephadex LH-20 with MeOH) to afford compound **1** (6 mg), **2** (15 mg), **3** (12 mg), **4** (35 mg), **5** (6 mg), **6** (30 mg) and **7** (7 mg). Fraction 2 (29 g) was chromatographed on Sephadex LH-20 and silica gel with petroleum:EtOAc (3:1) to yield compound **8** (21 mg), **9** (30 mg), **10** (80 mg) and **11** (15 mg).

3,3-Dimethoxy-24-ethyl-cholestan (1)

Colorless needles (MeOH); 1.2 millionth; $C_{31}H_{56}O_2$; mp: 81-82 °C; $[\alpha]_D^{24}$ +21.91° ($CHCl_3$, *c* 0.25); UV ($CHCl_3$) λ_{max}/nm (log ε): 252 (1.04); IR (KBr) ν 2950, 2870, 1463, 1382, 1332, 1175, 1136, 1111, 1049, 929, 873 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.19 (3H, s, H-30), 3.14 (3H, s, H-31), 1.95 (1H, m, H-12a), 1.86 (2H, m, H-16), 1.64 (1H, m, H-25), 1.63 (1H, m, H-7a), 1.60 (1H, m, H-8), 1.55 (1H, m, H-1a), 1.45 (1H, dd, *J* = 2.8, 14.8 Hz, H-2a), 1.43 (1H, m, H-6a), 1.32 (2H, m, H-4), 1.31 (1H, m, H-2b), 1.30 (1H, m, H-5), 1.24 (1H, m, H-6b), 1.16 (2H, m, H-23), 1.10 (1H, t, H-12b), 1.08 (1H, m, H-14), 1.07 (1H, m, H-1b), 0.99 (1H, m, H-17), 0.92 (1H, m, H-7b), 0.91 (1H, m, H-24), 0.90 (3H, d, *J* = 5.2 Hz, H-21), 0.84 (3H, t, *J* = 6.4 Hz, H-29), 0.82 (3H, d, *J* = 5.6 Hz, H-26), 0.80 (3H, d, *J* = 5.6 Hz, H-27), 0.70 (1H, m, H-9), 0.78 (3H, s, H-19), 0.64 (3H, s, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data see Table 1; EIMS *m/z* (%): 460 $[M]^+$ (2), 429 (12), 399 (28), 344 (14), 329 (8), 273 (4), 246 (7), 231 (70), 217 (30), 189 (9), 163 (30), 149 (52), 123 (57), 95 (63), 69 (53); HRESIMS *m/z*: 483.4164 (calcd. for $C_{31}H_{56}O_2Na$, 483.4178).

24α-Ethyl-cholestan-3α-ol (2)

Colorless needles ($CHCl_3$); 3.0 millionth; $C_{29}H_{52}O$; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 66.6 (d, C-3), 56.5 (d, C-14), 54.3 (d, C-9), 45.8 (d, C-24), 42.6 (s, C-13), 40.0 (t, C-12), 39.1 (d, C-5), 36.1 (d, C-20), 36.0 (s, C-10), 35.9 (t, C-4), 35.5 (d, C-8), 33.9 (t, C-22), 32.2 (t, C-1), 32.0 (t, C-7), 29.1 (d, C-25), 29.0 (t, C-2), 28.6 (t, C-6), 28.2 (t, C-16), 26.0 (t, C-23), 24.2 (t, C-15), 23.0 (t, C-28), 20.7 (t, C-11), 19.8 (q, C-26), 19.0 (q, C-27), 18.7 (q, C-21), 12.1 (q, C-18), 12.0 (q, C-29), 11.2 (q, C-19); EIMS *m/z* (%): 416 $[M]^+$ (100), 401 (50), 383 (44), 316 (8), 290 (18), 248 (25), 233 (64), 215 (57), 165 (45), 107 (47), 83 (26), 69 (33).

5β-24S-Ethylcholestan-3β-ol (3)

Colorless needles ($CHCl_3$); 2.4 millionth; $C_{29}H_{52}O$; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.10 (1H, C-3), 0.64 (3H, s, H-18), 0.96 (3H, s, H-19), 0.90 (3H, d, *J* = 6.5 Hz, H-21), 0.82 (3H, d, *J* = 7.7 Hz, H-26), 0.80 (3H, d, *J* = 7.7 Hz, H-27), 0.85 (3H, t, *J* = 7.5 Hz, H-29); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 67.1 (d, C-3), 56.6 (d, C-14), 56.2 (d, C-17), 45.8 (d, C-24), 42.7 (s, C-13), 40.2 (t, C-12), 39.7 (d, C-9), 36.6 (d, C-5), 36.1 (d, C-20), 35.6 (d, C-8), 35.1 (s, C-10), 33.8 (t, C-22), 33.5 (t, C-4), 29.9 (t, C-1), 29.0 (d, C-25), 28.3 (t, C-16), 27.8 (t, C-2), 26.6 (t, C-7), 26.2 (t, C-6), 26.0 (t, C-15), 24.2 (t, C-22), 23.9 (q, C-19), 23.0 (t, C-28), 21.1 (t, C-11), 19.8 (q, C-27), 19.0 (q, C-26), 18.7 (q, C-21), 12.0 (q, C-18), 11.9 (q, C-29); EIMS *m/z* (%): 416 $[M]^+$ (32), 402 (13), 401 (36), 383 (22), 316 (7), 290 (7), 248 (8), 233 (71), 215 (100), 165 (19), 107 (67), 81 (64), 69 (44).

Stigmastan-3-one (5)

White amorphous powder; 1.2 millionth; $C_{29}H_{50}O$; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.03 (3H, s, H-19), 0.93 (3H, d, *J* = 6.5 Hz, H-21), 0.87 (3H, t, *J* = 7.6 Hz, H-29), 0.86 (3H, d, *J* = 7.2 Hz, H-26), 0.84 (3H, d, *J* = 6.8 Hz, H-27), 0.70 (3H, s, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data see Table 1; EIMS *m/z* (%): 414 $[M]^+$ (24), 399 (6), 317 (4), 273 (4), 246 (8), 231 (100), 217 (37), 163 (28), 149 (29), 123 (36), 95 (40).

15-Nonacosanol (6)

White needles ($CHCl_3$); 6.0 millionth; $C_{29}H_{60}O$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.81 (6H, t, H-1, 29), 1.36 (4H, m, H-14, 16), 3.50 (1H, m, H-15); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.0 (q, C-1, 29), 22.5 (t, C-2, 28), 25.5 (t, C-13, 17), 31.80 (t, C-3, 27), 37.1 (t, C-14, 16), 71.7 (d, C-15); EIMS *m/z* (%): 424 $[M]^+$ (3), 423 (5), 297 (37), 278 (5), 195 (4), 181 (5), 157 (65).

Octyl palmitate (7)

White amorphous powder; 1.4 millionth; $C_{24}H_{48}O$; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.87 (6H, t, H-16,8′), 2.28

(2H, t, H-2), 4.05 (2H, t, H-1′); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 14.1 (q, C-16,8′), 22.7 (t, C-15, 7′), 25.0 (t, C-3), 28.6 (t, C-2′), 31.9 (t, C-14, 6′), 34.4 (t, C-2), 64.4 (t, C-1′), 174.1 (s, C-1); EIMS *m/z* (%): 368 $[M]^+$ (3), 354 (3), 341 (21), 313 (7), 257 (100), 256 (22), 239 (6), 153 (5), 125 (7).

20(29)-Lupene-3,28-diol (8)

White needles ($CHCl_3$); 4.2 millionth; $C_{30}H_{50}O_2$; ^{1}H-NMR (400 MHz, CD_3OD) δ 0.60 (3H, s, H-24), 0.68 (3H, s, H-25), 0.83 (3H, s, H-23), 0.85 (3H, s, H-26), 0.88 (3H, s, H-27), 1.80 (3H, s, H-30), 3.05 (1H, t, H-3), 3.12 (1H, d, *J* = 10.9 Hz, H-28a), 3.60 (1H, d, *J* = 10.9 Hz, H-28b), 4.42 (1H, s, H-29a), 4.52 (1H, s, H-29b); ^{13}C-NMR (100 MHz, CD_3OD) δ 38.5 (t, C-1), 26.6 (t, C-2), 78.4 (d, C-3), 38.4 (s, C-4), 55.1 (d, C-5), 18.0 (t, C-6), 34.0 (t, C-7), 40.6 (s, C-8), 50.2 (d, C-9), 36.8 (s, C-10), 20.5 (t, C-11), 25.0 (t, C-12), 37.0 (d, C-13), 42.4 (s, C-14), 26.5 (t, C-15), 28.9 (t, C-16), 47.4 (s, C-17), 47.5 (d, C-18), 48.5 (d, C-19), 150.4 (s, C-20), 29.4 (t, C-21), 33.6 (t, C-22), 27.5 (q, C-23), 15.0 (q, C-24), 15.6 (q, C-25), 15.7 (q, C-26), 14.4 (q, C-27), 59.5 (t,C-28), 109.2 (t, C-29), 18.7 (q, C-30); EIMS *m/z* (%): 442 $[M]^+$ (9), 427 (6), 412 (8), 411 (28), 385 (5), 234 (22), 220 (20), 207 (68), 203 (73), 189 (100), 175 (42), 135 (73), 95 (71), 81 (52).

Vanillin (9)

Yellow needles ($CHCl_3$); 6.0 millionth $C_8H_8O_3$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.95 (3H, s, OCH_3), 7.03 (1H, dd, *J* = 1.2, 8.4 Hz, H-6′), 7.79 (1H, d, *J* = 8.4 Hz, H-5′), 7.43 (1H, d, *J* = 1.2 Hz, H-2′), 9.82 (1H, s, CHO); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 56.0 (q, OCH_3), 108.9 (d, C-2′), 114.4 (d, C-5′), 127.5 (d, C-6′), 132.3 (s, C-1′), 147.2 (s, C-3′), 151.8 (s, C-4′), 191.0 (d, CHO); EIMS *m/z* (%): 152 ($[M]^+$, 93), 151 (100), 149 (40), 123 (20), 109 (17), 93 (14), 81 (28), 69 (41).

Acetovanillone (10)

Yellow needles ($CHCl_3$); 16 millionth; $C_9H_{10}O_3$; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.93 (3H, s, H-2), 7.32 (1H, d, *J* = 8.6 Hz, H-5′), 7.90 (1H, d, *J* = 2.1 Hz, H-2′), 7.91 (1H, dd, *J* = 2.1, 8.6 Hz, H-6′), 4.31 (3H, s, OCH_3); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 196.8 (s, C-1), 26.1 (q, C-2), 130.2 (s, C-1′), 109.8 (d, C-2′), 146.6 (s, C-3′), 150.4 (s, C-4′), 113.8 (d, C-5′), 124.0 (d, C-6′), 56.1 (q, OCH_3); EIMS *m/z* (%): 166 ($[M]^+$, 49), 151 (100), 123 (26), 85 (18).

Coniferaldehyde (11)

Yellow needles ($CHCl_3$); 3.0 millionth; $C_{10}H_{10}O_3$; ^{1}H-NMR (500 MHz, CD_3OD) δ 3.91 (3H, s, $OC\underline{H}_3$), 7.24 (1H, d, *J* = 1.8 Hz, H-2′), 7.16 (1H, dd, *J* = 8.2, 1.8 Hz, H-6′), 6.84 (1H, d, *J* = 8.2 Hz, H-5′), 9.56 (1H, d, *J* = 7.9 Hz, H-1), 7.57 (1H, d, *J* = 15.7 Hz, H-3), 6.64 (1H, dd, *J* = 15.7, 7.9 Hz, H-2); ^{13}C-NMR (125 MHz, CD_3OD) δ 56.5 (q, OCH_3), 192.9 (d, C-1), 156.2 (d, C-3), 151.7 (s, C-4′), 149.5 (s, C-3′), 127.9 (d, C-2), 127.8 (s, C-1′), 116.7 (d, C-5′), 125.1 (d, C-6′), 112.3 (d, C-2′); EIMS *m/z* (%): 178 ($[M]^+$, 100), 177 (29), 163 (11), 161 (20), 135 (25), 107 (18).

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